Impact of preimplantational oral low-dose estradiol-17 exposure on the endometrium: The role of miRNA

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Abstract: Porcine conceptuses synthesize estrogens between Day 11 and 12 as signal for maternal recognition of pregnancy. A preimplantational estrogen exposure to pregnant gilts has been associated with embryonic losses and changes in endometrial mRNA expression. MicroRNAs (miRNAs) play a key role in the mRNA regulation by modulating the expression. Effects of estrogens on endometrial miRNAs have not been investigated in this context so far. Thus, we studied the endometrial expression profile of miRNAs in the pig at gestational Day 10 after daily estradiol-17 (E2) application starting at fertilization using either 0, 0.05 (ADI-acceptable daily intake), 10 (NOEL-no-observed-effect level) and 1,000 (high dose) µg E2/kg body weight/day, respectively. In endometrial homogenates, E2 ($p < 0.001$) and total estrogen concentrations ($p < 0.001$) were significantly increased, namely 28- and 160-fold, respectively, in the high dose group as compared to the control. Additionally, total estrogens were sixfold elevated in the NOEL group. Interestingly, high-throughput sequencing of small non-coding RNA libraries did not indicate any differentially expressed miRNAs between the treatment groups and the control group. The expression of 12 potential E2 target miRNAs investigated by RT-qPCR were equally unaffected. Thus, preimplantational E2 exposure resulted in significantly higher endometrial estrogen concentrations, but did not perturb the expression profile of endometrial miRNAs.

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RESEARCH ARTICLE

Impact of preimplantational oral low-dose estradiol-17β exposure on the endometrium: the role of miRNA†

Short title: E2 effects on endometrial miRNA.

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Abbreviations: ADI, acceptable daily intake; EDCs, endocrine disrupting chemicals; E2, estradiol-17β; ER, estrogen receptor; miRNAs, microRNAs; ncRNAs, non-coding RNAs; NOEL, no-observed-effect level.

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SUMMARY

Porcine conceptuses synthesize estrogens between day 11 and 12 as signal for maternal recognition of pregnancy. A preimplantational estrogen exposure to pregnant gilts has been associated with embryonic losses and changes in endometrial mRNA expression. MicroRNAs (miRNAs) play a key role in the mRNA regulation by modulating the expression. Effects of estrogens on endometrial miRNAs have not been investigated in this context so far. Thus, we studied the endometrial expression profile of miRNAs in the pig at gestational day 10 after daily estradiol-17β (E2) application starting at fertilization using either 0, 0.05 (ADI – acceptable daily intake), 10 (NOEL – no-observed-effect level) and 1000 (high dose) µg E2/kg body weight/day, respectively. In endometrial homogenates, E2 ($p < 0.001$) and total estrogen concentrations ($p < 0.001$) were significantly increased, namely 28- and 160-fold, respectively, in the high dose group as compared to the control. Additionally, total estrogens were 6-fold elevated in the NOEL group. Interestingly, high-throughput sequencing of small non-coding RNA libraries did not indicate any differentially expressed miRNAs between the treatment groups and the control group. The expression of 12 potential E2 target miRNAs investigated by RT-qPCR were equally unaffected. Thus, preimplantational E2 exposure resulted in significantly higher endometrial estrogen concentrations, but did not perturb the expression profile of endometrial miRNAs.

Keywords: Estrogen; Pig; Pregnancy; Deep sequencing; Endocrine disrupting chemicals

INTRODUCTION

Endocrine disrupting chemicals (EDCs) are a class of exogenous substances affecting endogenous hormonal systems, reproduction and health (Diamanti-Kandarakis et al., 2009). Estradiol-17β (E2) can exhibit properties of an EDC (Pope et al., 1986; Geisert et al., 1991; Malcolm et al., 2006; Rasier et al., 2006). Suitably, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) announced an acceptable daily intake (ADI) of 0.05 µg E2 /kg body weight (bw) for humans in 1999 (JECFA, 1999). Only recently, we demonstrated that orally administered low doses of E2 to pigs during the entire period of pregnancy affected body composition in male offspring (Fürst et al., 2012), bone parameters in male and female offspring (Flöter et al., 2016), as well as gene expression in the prostate (Kradolfer et al., 2016). Especially prenatal development, starting as early as the preimplantation phase, has been demonstrated to be a sensitive period, when disruptive stimuli may induce long-term consequences (Ma et al., 2003; Amstislavsky et al., 2004; Amstislavsky et al., 2006; Diamanti-Kandarakis et al., 2009; Hochberg et al., 2011). Particularly in the pig, effects as strong as embryonic losses have been observed (Pope et al., 1986; Geisert et al., 1991). Such effects may arise through changes in the endometrial gene expression and secretion (Geisert et al., 2006). Evidence is increasing that small non-coding RNAs (ncRNAs), especially microRNAs (miRNAs), are important gene regulatory molecules in the endometrium (Chegini, 2010; Bidarimath et al., 2014). In addition, the mechanisms of miRNAs altering mRNA expression are well studied and it has been predicted that miRNAs regulate about 50 % of all mammalian protein-coding genes (Krol et al., 2010; Zhou et al., 2010; Roberts, 2014). Still, there is especially little knowledge about effects on miRNA expression through EDCs during the early stages of development (Nothnick and Healy, 2010; Meunier et al., 2012; Veiga-Lopez et al., 2013; Felice et al., 2015).

MicroRNAs are involved in the regulation of various processes such as apoptosis, cell proliferation and development (Bartel, 2004). Expression profiling has identified a large number of miRNAs in reproductive tissues, including the ovary and the endometrium

(Chegini, 2010; Su et al., 2014). Endometrial miRNAs have been associated with the regulation of genes that are important for differentiation, proliferations, receptivity, extracellular matrix factors, angiogenesis and immunological response processes, which are crucial in preparation of the endometrium for implantation (Wessels et al., 2013; Bidarimath et al., 2014; Krawczynski and Bauersachs et al., 2015). Steroid hormones and their respective receptors such as estrogen receptors (ER) α and β largely control these processes (Chegini, 2010). Information about estrogen regulated expression of miRNAs comes primarily from research in breast cancer cell lines treated with E2 (Cicatiello et al., 2010; Klinge, 2012). Moreover, the potential regulation of endometrial miRNAs by ovarian steroids was indicated through their different expression during the menstrual cycle (Chegini, 2010; Sha et al., 2011), as well as through treatment experiments (Nothnick and Healy, 2010). Alterations in miRNA expression were observed after E2 treatment of human endometrial stromal and glandular epithelial cells (Toloubeydokhti et al., 2008). Nothnick et al. (Nothnick and Healy, 2010) demonstrated the involvement of the classical pathway of estrogens acting through ERα, as after pretreatment with the ERα specific inhibitor ICI 182,780, E2 did not alter the miRNA expression.

In the present study, we aimed at elucidating if continuous preimplantational E2 exposure affects the expression of endometrial miRNAs. Therefore, we analyzed the influence of three distinct concentrations of E2 – the dose corresponding to the ADI, a dose close to the no-observed-effect level (NOEL) and a high dose – on the endometrial expression profile of miRNAs in sows at day 10 of pregnancy.

RESULTS

Evaluation of the Embryos

All recovered embryos (n = 230) were hatched blastocysts and showed normal, stage-specific development and displayed an embryonic disc. Neither earlier stages (2-, 4-, 6-, or 8-cell-stage embryos) nor elongated embryos were observed. There was neither significant difference between the treatment groups and the control group in the number of embryos ($p = 0.33$) nor in the size of the embryos ($p = 0.80$) with 1.89 mm ± 0.48 mm (n = 61), 2.48 mm ± 0.55 mm (n = 54), 1.91 mm ± 0.43 mm (n = 61) and 2.27 mm ± 0.48 mm (n = 54) in the control, ADI, NOEL and the high dose group, respectively.

Abundance of Estrogens in Endometrial Samples

The concentration of endometrial E2 was significantly affected by the oral E2 treatment (overall $p < 0.001$; Fig. 1). E2 depicted higher amounts in the high dose group compared to the control group (3056 ± 830 pg/g and 111 ± 4 pg/g, respectively). E2 in neither the ADI (82 ± 22 pg/g) nor the NOEL group (195 ± 57 pg/g) was significantly different from the control group. The abundance of total endometrial estrogens (estrone, E2, estradiol-17α), which in the pig is the sum of E2 and estrone (E1) due to negligible amounts of estradiol-17α, was also significantly affected (overall $p < 0.001$; Fig. 1). Total estrogens were unaltered in the ADI group (156 ± 22 pg/g), but higher in the NOEL (881 ± 252 pg/g) and the high dose group (24,657 ± 9,557 pg/g) in comparison to the control group (154 ± 12 pg/g).

Measurement of miRNA Expression with High-Throughput Sequencing

Small ncRNA sequencing of porcine endometrium was performed. The number of raw reads per library and the number of discarded reads as yielded in the following quality control steps are detailed in Table S1. The relative frequencies are shown in Figure 2A. The number of raw reads as well as the number of total reads kept were similar between the treatment groups ($p > 0.05$). There was an average of 13.0 million ± 1.2 million raw reads (mean ±

SEM; n = 16). The selection of good quality reads from the raw reads resulted in an average of 9.2 million ± 0.8 million reads (mean ± SEM; n = 16) corresponding to 71 % of the total reads (Fig. 2A, Table S1).

The quality of the kept reads was comparable in all 16 libraries as indicated by the analysis with FastQC. The good quality of the reads was shown by a mean Phred score of mainly 38 (Fig. 2B). The majority of the reads, derived from the FastQC report, had a length of 21 to 23 nucleotides (Fig. 2C). This indicates that the samples mainly contained miRNAs.

After the sequences were counted and filtered to remove very low abundant transcripts, there were in total 41,503 different sequences left, assumingly containing various kinds of small ncRNAs. In the database, there were 326 annotated mature miRNAs in the pig (miRBase 20.0). As miRNAs are conserved between species, the data were also compared to the miRNA sequences from miRBase of human (2578 annotated miRNAs), cow (783 annotated miRNAs) and mouse (1908 annotated miRNAs). In the endometrial samples, 212 porcine, 272 human, 205 bovine and 235 murine known mature miRNAs were detected. These detected mature miRNAs were analyzed per species with the DESeq algorithm. The expression of these mature miRNAs did not differ significantly (adjusted $p > 0.05$) between the treatment groups and the control group in any of the species analyzed. The 20 highest expressed miRNAs representing 82.7 % of all reads of the 326 porcine mature miRNAs are shown in Figure 3A. The miR-21 was highest expressed with 12.6 % and the remaining 192 porcine miRNAs accounted for 17.3 % of the reads. The cluster analysis for the 30 porcine miRNAs with the highest variance revealed that the individual samples did not cluster related to the treatment group (Fig. 3B). The lack of clustering emphasizes that there was no effect of the treatment on the miRNA expression.

miRNAs are quite conserved across species, therefore, many miRNA sequences found in the endometrial samples are annotated in more than one species (Fig. 4). As shown in Figure 4, about one third of the known 212 porcine miRNAs are only found in the pig (n = 62), while about another third (n = 63) matched to known miRNAs from all four species. Overall, the detected 212 porcine, 272 human, 205 bovine and 235 murine sequences

resulted without this overlap in a total of 444 expressed mature miRNA sequences in the porcine endometrium. Thus, in addition to the 212 porcine sequences (47.7 %), a further 232 sequences were found matching 100 % to known miRNAs from the human, cow and/or mouse (Fig. S1) most likely corresponding to not yet annotated porcine miRNAs and miRNA variants. A table of these sequences including the matching miRNA information from the four species can be found in Table S2.

Measurement of miRNA Expression with RT-qPCR

Twelve potentially E2 regulated miRNAs were selected for the miRNA expression analysis using RT-qPCR. All of these miRNAs have been described in the literature in the context of altered expression due to an E2 exposition (Pan et al., 2007; Cohen et al., 2008; Bhat-Nakshatri et al., 2009; Klinge, 2009; Maillot et al., 2009; Wickramasinghe et al., 2009; Wang et al., 2010; Katchy et al., 2012; Di Leva et al., 2013; Zhao et al., 2013). The miR-20a and miR-21 have been repeatedly shown to be altered upon E2 treatment (Pan et al., 2007; Klinge, 2009; Wickramasinghe et al., 2009; Wang et al., 2010; Klinge, 2012). The other candidate miRNAs were selected related to different absolute expression abundance, namely from about 100 reads (miR15a, miR-29c, miR-130a), about 1000 reads (miR-16, miR-20a, miR-146, miR-195, miR-205), about 10,000 reads (miR-27b, miR103a, miR-191) up to about 100,000 reads (miR-21). Particularly, in the breast cancer cells MCF-7 and T47D, miR-15a, miR-16, miR-20a, miR-21, miR-27b, miR-29c, miR-103, miR-146b, miR-191 and miR-195 have been shown to be influenced after E2 treatment (Bhat-Nakshatri et al., 2009; Klinge, 2009; Katchy et al., 2012; Di Leva et al., 2013). miR-20a and miR-21 have been additionally differentially expressed in human endometrial stroma and glandular cells, respectively, as well as in cancerous endometrium and endometriosis (Pan et al., 2007; Wickramasinghe et al., 2009; Wang et al., 2010). Furthermore, miR-205 has been upreguated by *ex vivo* E2 treatment of mouse aorta (Zhao et al., 2013) while miR-130a has been downregulated after E2 exposure in whole-body homogenates of zebrafish (Cohen et al., 2008). The expression of RNU6B, RNU5A and SNORA73A was used for the

normalization, as determined with the GeNorm and Normfinder algorithm. In all three E2 treatment groups, the endometrial expression of none of the 12 target miRNAs displayed significant difference ($p > 0.05$) to the respective control group as shown in Table 2 and Figure S2. Most often, very similar expression values were observed. Only miR-146b showed a larger variance, but similarly no significant difference ($p = 0.62$).

DISCUSSION

The oral application of a high dose of E2 over the first 10 days of pregnancy lead to a 28-fold increase in endometrial E2 concentrations compared to the control group. In addition, endometrial total estrogens were not only pronouncedly elevated in the high dose group (160-fold), but also in the animals receiving the NOEL dose (6-fold). Fürst et al. (Fürst et al., 2012) demonstrated that the two low doses of E2, corresponding to the ADI and close to the NOEL, administered during the entire length of gestation, affected body weight development and body composition, respectively, in the offspring. Further low-dose effects in the offspring were observed by analyzing the bone (Flöter et al., 2016) and prostate (Kradolfer et al., 2016). These lasting effects may be due to the altered endocrine environment during pregnancy, particularly due to exposure effects already occurring during the time of preimplantation when still low endogenous estrogen concentrations prevail. For this reason, the present *in vivo* study focused on analyzing day 10 after fertilization, shortly prior to the endogenous estrogen signal secreted by the porcine embryo. The high dose group depicted a mean concentration of 3.1 ng E2/g endometrial tissue compared to 0.1 ng E2/g in the control animals, resulting from accumulated and/or remaining E2 over 10 days through feeding of 500 µg E2/kg bw twice per day (Fürst et al., 2012) and through timing of the slaughtering one hour after feeding the regular dose of 500 µg E2/kg bw. This is a time, where still high concentrations of E2 can be observed in the peripheral blood (Fürst et al., 2012). Using high-throughput ncRNA sequencing, 444 sequences of mature miRNAs were

detected, however, not differentially expressed between the treatment groups and the control. This was further confirmed by RT-qPCR of 12 selected, potentially E2 dependent miRNAs (Pan et al., 2007; Cohen et al., 2008; Bhat-Nakshatri et al., 2009; Klinge, 2009; Maillot et al., 2009; Wickramasinghe et al., 2009; Wang et al., 2010; Katchy et al., 2012; Di Leva et al., 2013; Zhao et al., 2013). In many cell culture experiments, most often using MCF-7 human breast cancer cells ,10 nM E2 (2.7 ng E2/ml) was applied as treatment dose (Klinge, 2012). This lead to alterations in the expression of miRNAs as reviewed by Klinge (Klinge, 2012) including miR-15a, miR-16, miR20a, miR21, miR-27b, miR-103, miR146b, miR191 and miR-195 (Bhat-Nakshatri et al., 2009; Klinge, 2009; Maillot et al., 2009; Di Leva et al., 2013), which were also determined in the present study using RT-qPCR. The difference in E2 responsiveness in the latter studies compared to the study at hand may have its origin in the biological background, as breast cancer cell lines are out of the biological context and characterized by exceptional high concentrations of ERα (Klinge, 2012).

In contrast to miRNAs, differential endometrial gene expression has been observed on the mRNA level in the same samples in all three treatment groups (Flöter et al., under review). There were 14 (ADI), 17 (NOEL) and 27 (high dose) differentially expressed genes (DEG) in the endometrium with at least a 2-fold regulation. An earlier study by Ross et al. (Ross et al., 2007) injected intramuscularly estradiol cypionate to sows only at days 9 and 10 of pregnancy and observed 9 (day 10), 71 (day 13) and 21 (day 15) DEG with at least 1.8-fold regulation. Interestingly, some DEG were upregulated in both studies. In the high dose group *RBP4* (retinol binding protein 4), which was also altered at day 10 (Ross et al., 2007), and *SULT2A1* (sulfotransferase family, cytosolic, 2A, dehydroepiandrosterone (DHEA)-preferring, member 1), *VNN2* (vanin 2) as well as *SLC39A2* (solute carrier family 39 (zinc transporter), member 2), which were altered at day 13 (Ross et al., 2007), were elevated. Thus, it is even more surprising that miRNAs in our study were unaffected, as they are known important regulators of mRNA expression.

The most important aspect seems to be the timing of the estrogen exposure, which is different in the study at hand compared to most studies available in the pig. Not only altered endometrial expression of genes had been observed after exposure at days 9 and/or 10 of pregnancy, but also degeneration of the uterine glycocalyx (Ross et al., 2007) and even embryonic losses (Pope et al., 1986; Geisert et al., 1991). Thus, estrogen exposure shortly before the embryo starts secreting estrogens as embryonic signal for maternal pregnancy recognition which occurs at days 11 to 12 (Spencer et al., 2004) can exert major impact. In contrast, neither estrogen exposure at days 12 and 13 (Pope et al., 1986) nor only at day 12 (Geisert et al., 1991) induced embryonic losses. Thus, there is a strong time-point dependent component regarding effects through estrogens during the periimplantational phase. Molecular analyses indicate that exogenous estrogen may lead to a desynchronization of endometrial receptivity and embryo development resulting in embryonic loss (Geisert et al., 2006). There are scarce reports about studies which administered estrogens already starting at insemination. Evidence exists in “imprinting control region” (ICR) mice, a multipurpose research mouse model strain (Okada and Kai, 2008). Female ICR mice were implanted with an E2-containing tube prior to mating, which was maintained during pregnancy and lactation. Interestingly, most of the animals maintained their pregnancy and had a birth rate of 70 %. Our continuous treatment neither lead to a change in the number of embryos at day 10 (this study) nor in litter size (Fürst et al., 2012). Thus, we might observe a habituation or programming with subtle, but not detrimental effects towards the presence of estrogens. Certain alterations were observed in the pre- and postpubertal offspring (Fürst et al., 2012; Flöter et al., 2016; Kradolfer et al., 2016), which might also result from exposure during this very early time of development. Additionally, this leads to the possibility that administering estrogens on days 9 and 10 of pregnancy, might lead to changes in miRNA expression particularly at days 12 to 15 possibly involved in the drastic outcome of abortion (Pope et al., 1986; Geisert et al., 1991).

Many studies have demonstrated effects of an exposure to EDCs during embryo development directly on mothers and offspring as well as long-term effects in the latter (Ma

et al., 2003; Amstislavsky et al., 2004; Amstislavsky et al., 2006; Geisert et al., 2006; Ross et al., 2007; Gore et al., 2015). Little is known about EDCs affecting miRNAs (Nothnick and Healy, 2010; Meunier et al., 2012; Veiga-Lopez et al., 2013; Felice et al., 2015). A former study has demonstrated direct *in utero* effects of bisphenol A altering fetal ovarian miRNA expression in sheep (Veiga-Lopez et al., 2013). A further study showed lasting changes after neonatal exposure to estradiol benzoate on adult rat testes including alterations in miRNA expression (Meunier et al., 2012). In contrast, there was no E2 effect on the miRNA profile in the present study, although endometrial as well as embryonic mRNA changes showed E2-dependent differentially expressed transcripts (Flöter et al., under review). Several reasons can account for this striking finding: the window of exposure, the route of administration, the substance-specific elimination kinetics, the continuous treatment that could have induced a habituation towards estrogens, as well as the timing of the analysis one hour after the last dose was fed. Other mechanisms of gene expression regulation than differential miRNA expression may as well account for the observed differential mRNA expression. Further functional studies need to be undertaken.

In summary, continuously applied E2 to pregnant pigs by oral ingestion increased endometrial estrogen concentrations even at a low dose that is close to the announced NOEL for humans (JECFA, 1999). Although local estrogen concentrations increased pronouncedly and lead to a perturbed mRNA expression (Flöter et al., under review), there was no E2 treatment effect on the expression profile of miRNAs in the endometrium during the blastocyst stage of pregnancy. As miRNAs can be found in extracellular vesicles (EV) from uterine flushings (Krawczynski and Najmula et al., 2015), the miRNA load of EV remains an important target for future analyses of periconceptional effects of EDCs.

MATERIALS AND METHODS

Animal Studies and Collection of Tissue Samples

In order to investigate effects of E2 on the expression profile of miRNAs in the pregnant endometrium, the present study was undertaken according to our previous study applying E2 during the entire pregnancy (Pistek et al., 2013). In brief, the estrous cycles of the German Landrace sows were synchronized prior to the start of the treatment. The sows were inseminated with the sperm of one Pietrain boar. For the E2 exposure, the sows were randomly assigned to a treatment group (n = 5-6 per group). One group received 0.05 µg E2/kg bw/day, corresponding to the announced ADI for humans, while another group obtained 10 µg E2/kg bw/day, related to the NOEL (JECFA, 1999). In addition, a high dose of 1000 µg E2/kg bw/day and ethanol carrier only for the control group were used, respectively. Half of the E2 dose, dissolved in 2 ml ethanol, was fed via bread rolls (20 g) in the morning and the other half in the evening. The E2 was applied continuously for a period of 10 days, beginning with insemination until slaughter at day 10 of pregnancy. The last dose was fed one hour before slaughter. The uterus was flushed with 10 ml and another 50 ml of phosphate-buffered saline (PBS, autoclaved, pH 7.4) to retrieve the embryos. All embryos were collected in a petri-dish with PBS. Subsequent, pictures of the embryos were taken. After carefully opening the uterus, endometrial samples were collected, shock frozen in liquid nitrogen and stored at – 80 °C. Plasma samples were retrieved after centrifugation of EDTA supplemented blood at 4 °C and stored at – 20 °C. For the small ncRNA sequencing analyses, only animals with embryos at the blastocyst stage (n = 3-5 per group) were considered. Three sows did not conceive, as only unfertilized oocytes were found (one animal each in the control, ADI and high dose group, respectively). In addition, two animals of the ADI dose group were excluded from the study due to illnesses at slaughtering. Both animals had pus and clinical signs of inflammation in the uterus and had not been pregnant. The experiments were conducted with permission from the local veterinary authorities and were performed in accordance with the accepted standards of humane animal care.

Hormone Analyses

Analyses of estradiol-17β (E2) and total estrogen (estrone, E2, estradiol-17α) concentrations in endometrial tissue were performed using an in-house competitive enzyme immuno assays (EIA) (Meyer et al., 1990; Hageleit et al., 2000). Endometrial tissue was homogenized in liquid nitrogen using a mortar and pestle. Saline (0.5 ml) was added to 100 mg of the grounded tissue. For the extraction, the tissue was at first incubated in 6.5 ml tert. butylmethylether/petrolether 30/70 v/v overnight. After phase separation at room temperature within two days, it was frozen at – 60 ºC for 48 hours. The liquid ether phase was decanted and the ether was vaporized. After adding of 500 µl assay puffer, abundance of E2 and total estrogens was determined as described earlier (Hageleit et al., 2000).

Extraction and Quality of RNA

For further analyses, isolation of total endometrial RNA from the collected samples was performed by means of TRIzol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer´s protocol (n = 4 per group, except for the ADI group, where a repeated tissue extraction was done because of only n = 3 available animals). For determining the purity and the quantity of the obtained RNA, the NanoDrop 1000 (peqLab, Erlangen, Germany) was applied. The RNA integrity was measured with a Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany). The mean RNA integrity number (RIN) was 9.4 ± 0.4 (± SD).

Small ncRNA Sequencing of Endometrial Samples

Total RNA was used for high-throughput sequencing, which was kindly performed by the Genomics Core Facility of the EMBL Heidelberg (EMBL Heidelberg, Genomics Core Facility, Heidelberg, Germany). For preparation of the small RNA-Seq libraries, the NEBNext© Small RNA Sample Prep Set 1 (New England Biolabs, Frankfurt-Höchst, Germany) was utilized

according to the manufacturer´s instructions. The sequencing was conducted on an Illumina HiSeq 2000 (San Diego, USA) with single-read mode and a read length of 50 bases.

Analysis of the Illumina Sequence Data with Galaxy

The Fastq files were analyzed with a locally installed version of Galaxy (Giardine et al., 2005) (www.usegalaxy.org, hosted by Gene Center Munich, AG Blum). The reads were trimmed from the 5´and 3´end with “Fastq quality trimmer” (Blankenberg et al., 2010) (window size: 3, step size: 1, quality score ≥ 30.0, aggregate action for window: mean of scores), the adapter sequence was clipped (min. sequence length: 17, discard sequences with unknown (N) bases: yes) and reads filtered with “filter by quality” (quality cut-off value: 25, percentage of bases in the sequence that must have a quality of at least the cut-off value: 100). Retrained reads were used for the quality report with FastQC. Subsequently, the abundance of each unique sequence was determined, the table of the read counts from all samples was then filtered to remove very low abundant sequences and to keep potential transcripts that are turned on or off by the E2 treatment (condition: at least 10 reads of each miRNA must be present in three to four samples of at least one group). As miRNAs are quite conserved between species, our data were also compared to the miRNA sequences of human, cow and mouse. Therefore, databases of mature miRNAs for pig (326 annotated miRNAs), human (2578 annotated miRNAs), mouse (1908 annotated miRNAs) and cow (783 annotated miRNAs) were obtained from miRBase (www.miRBase.org, release 20.0) and used to generate a BLAST database in the Galaxy platform utilizing “make blast database” (http://www.ncbi.nlm.nih.gov/books/NBK1763/) (molecule type of input: nucleotide, hash-index: true). Reads were mapped against these databases with “blastn” (Zhang et al., 2000) (blastn-short, expectation value: 1.0, word size: 5), followed by filtering of the blast output (condition: length of query sequence = length of target sequence = alignment length and number of identical bases within the alignment) in order to selected only miRNAs which aligned 100 % with the database sequence.

NGS experiments have been deposited in NCBI's Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo) repository with accession number GSE89343.

Quantitative Real-Time qPCR

The RNA samples were reverse transcribed into cDNA using miScript II RT kit (Qiagen, Hilden, Germany) according to the recommendations of the manufacturer. Defined cDNA fragments were amplified by quantitative real-time PCR (RT-qPCR) with specific forward primers (miScript Primer Assay, Qiagen) (Table 1). All primers are specific for the pig. If the porcine (ssc) miRNA sequence was identical with the mouse (mmu) or human (hsa) sequence, these existing primers were used. Twelve potential E2 regulated miRNAs were selected for RT-qPCR validation (Pan et al., 2007; Cohen et al., 2008; Bhat-Nakshatri et al., 2009; Klinge, 2009; Maillot et al., 2009; Wickramasinghe et al., 2009; Wang et al., 2010; Katchy et al., 2012; Di Leva et al., 2013; Zhao et al., 2013). As potential reference genes the human miScript Control Assays (Qiagen) for small nuclear RNAs (snRNA) RNU6B (NR 002752.2) and RNU5A (NR 002756.2), and the small nucleolar RNAs (snoRNA) SNORA73A (NR 002907.2), SNORA25 (NR 003028.1) and SCARNA17 (NR 003003.2) were additionally determined.

The RT-qPCR reaction was performed with the Rotor-Gene (Qiagen) using the QuantiTect SYBR Green PCR kit (Qiagen). The master mix had a final volume of 10 µl consisting of 1 µl miScript Primer Assay, 1 µl miScript Universal Primer, 5 µl QuantiTect SYBR Green PCR Master Mix and 3 µl of 1:4 diluted cDNA. For the negative control, nuclease free water instead of cDNA was used. In all assays, standard cycling conditions were as followed: 95 °C for 15 min, then 40 cycles of 94 °C for 15 s, 55 °C for 30 s and 70 °C for 30 s, and a melting curve. The cycle of quantification (Cq) was calculated by the Rotor-Gene software (Rotor-Gene® Q Series 1.7; Qiagen). The selection of appropriate reference genes was based on the calculations from the GeNorm and Normfinder algorithm (GenEx software 3.4.3 (Gothenburg, Sweden). For the normalization process RNU6B, RNU5A, SNORA73A were

utilized. The resulting ΔCq-values were further analyzed with the ΔΔCq method (Livak and Schmittgen, 2001).

Statistical Analyses

For statistical analyses, regarding the RT-qPCR experiments the ΔCq-values were used. The logarithmized data of the hormone concentrations were taken for statistical analyses. The miRNA expression data, obtained by RT-qPCR, the endometrial hormone concentrations, measured by EIA, as well as the read counts and the number of embryos per sow were analyzed with one-way ANOVA followed by a Dunnetts´s *post hoc* test to evaluate potential differences of the treatment groups compared to the control group using the SigmaPlot program 11.0 (SPSS, Chicago, IL, USA). Embryo size was analyzed using SAS 9.2 (SAS Institute, Inc.,Cary, NC, USA) in order to account for the nested study design of multiple embryos belonging to the same exposed sow. A mixed model including the repeated measurement function for the embryos and a random intercept for the mother sows was applied as described by Kiernan and colleagues (Kiernan et al., 2012). The residual method was used to calculate the denominator degrees of freedom (Bell B.A. et al., 2013). The Dunnett's *post hoc* test was applied. The results from SAS are presented as mean ± SE. Regarding the data from the small ncRNA sequencing experiment, analysis of differential miRNA expression in a treatment group versus the control group was performed with DESeq 2.11 (Anders and Huber, 2010) (www.bioconductor.org) in R 2.15.3. Differences were considered significant at an adjusted p-value from the snRNA sequencing < 0.05 and in all other experiments at $p < 0.05$. Mean values ± SEM were used for graphical presentation of the statistical results.

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Figure Legends

Figure 1. Abundance of estradiol-17β (E2) and total estrogens in the porcine endometrium after E2 exposure. The three treatment groups corresponding to the acceptable daily intake (ADI), close to the NOEL (no-observed-effect level,) and a high dose (0.05, 10 and 1000 µg E2/kg body weight/day, respectively) were compared to the control animals. Significant differences are indicated by an asterisk ($p < 0.05$). Values are depicted as mean ± SEM (n = 4-6 per group).

Figure 2. Reads during quality assessment. A) Proportion of reads. The 16 samples (n = 4 per treatment group) are depicted on the x-axis. Each biological replicate is indicated by a different number following the treatment information (control, acceptable daily intake (ADI), no-observed-effect level (NOEL) and high dose corresponding to 0, 0.05, 10 and 1000 µg E2/kg bw/d). The raw reads correspond to 100 %. Reads were stepwise discarded. First, reads were discarded by trimming. Next, adaptor only reads, reads without the 3' adaptor, reads with unknown bases (N) and sequences shorter than 17 nucleotides were discarded depicted as „discarded reads by adaptor clipping". Then, reads containing bases with a Phred score below 25 were discarded "due to low quality". B) The mean Phred score per sequence (x-axis) of the "total reads kept" is shown for the four treatment groups with the respective number of sequences (y-axis). Most reads had a mean Phred score of 38, irrespective of the treatment group. C) Read length distribution of the "total reads kept" with mean values of all 16 samples. A peak can be observed at 21 to 23 nucleotides.

Figure 3. Annotated porcine endometrial miRNAs. A) The top 20 expressed porcine miRNAs with the percentage of read counts in addition to the sum of all remaining miRNAs are depicted. B) The heatmap of the 30 porcine miRNAs with the highest variance using rlog transformed data is shown, displaying similar expression in the samples and consequently no clustering according to the four treatment groups. Each biological replicate is indicated by

a different number following the treatment information (Control, acceptable daily intake (ADI), close to the no-observed-effect level (NOEL) and a high dose group (0, 0.05, 10 and 1000 µg/kg bw/d, respectively)). ssc, sus scrofa.

Figure 4. Distribution of the 444 detected sequences of mature miRNAs. The Venn diagram represents the number of detected sequences split into the species to which the sequence mapped to 100 % to a known miRNA. The diagram below depicts the number of mapped sequences per species. At the bottom, the number of sequences that are shared by 1, 2, 3 or 4 species is shown.

Table 1. miScript Primer Assays for 12 potential porcine target miRNAs

miRNA name	Mature miRNA sequence 5´ → 3´	MIMAT*
hsa-miR-15a-5p	UAGCAGCACAUAAUGGUUUGUG	68
hsa-miR-16-5p	UAGCAGCACGUAAAUAUUGGCG	69
mmu-miR-20a-5p	UAAAGUGCUUAUAGUGCAGGUAG	529
hsa-miR-21-5p	UAGCUUAUCAGACUGAUGUUGA	76
hsa-miR-27b-3p	UUCACAGUGGCUAAGUUCUGC	419
hsa-miR-29c-3p	UAGCACCAUUUGAAAUCGGUUA	681
hsa-miR-103a-3p	CAGUGCAAUGUUAAAAGGGCAU	425
hsa-miR-130a-3p	CAGUGCAAUGUUAAAAGGGCAU	425
ssc-miR-146b	UGAGAACUGAAUUCCAUAGGC	10190
hsa-miR-191-5p	CAACGGAAUCCCAAAAGCAGCUG	440
hsa-miR-195-5p	UAGCAGCACAGAAAUAUUGGC	461
hsa-miR-205-5p	UCCUUCAUUCCACCGGAGUCUG	266

* accession number (MIMAT) from miRBase

Table 2. Normalized expression of 12 endometrial miRNAs at day 10 of pregnancy after E2 exposure.

miRNA	**Control group** [Mean 40+ΔCq ± SEM]	**ADI dose group** [Mean 40+ΔCq ± SEM]	**NOEL dose group** [Mean 40+ΔCq ± SEM]	**High dose group** [Mean 40+ΔCq ± SEM]	**Overall p-value**
miR-15a	29.3 ± 0.7	29.1 ± 0.6	29.7 ± 0.3	29.5 ± 0.7	0.67
miR-16	35.7 ± 0.3	35.1 ± 0.3	35.6 ± 0.1	34.8 ± 0.3	0.10
miR-20a	31.0 ± 0.5	31.0 ± 0.6	31.0 ± 0.3	31.0 ± 0.6	1.00
miR-21	38.2 ± 0.5	37.8 ± 0.5	38.6 ± 0.2	37.9 ± 0.5	0.55
miR-27b	33.7 ± 0.4	33.8 ± 0.1	34.0 ± 0.4	33.8 ± 0.4	0.96
miR-29c	34.4 ± 0.6	34.3 ± 0.4	34.3 ± 0.3	34.6 ± 0.5	0.96
miR-103	32.3 ± 0.4	32.2 ± 0.2	32.5 ± 0.2	38.3 ± 0.4	0.54
miR-130a	30.3 ± 0.3	30.4 ± 0.3	30.2 ± 0.2	30.5 ± 0.4	0.94
miR-146b	25.8 ± 1.4	25.8 ± 1.6	23.4 ± 1.8	25.2 ± 0.9	0.62
miR-191	34.2 ± 0.2	33.8 ± 0.3	33.7 ± 0.2	33.8 ± 0.3	0.53
miR-195	35.1 ± 0.3	34.2 ± 0.5	35.2 ± 0.1	34.8 ± 0.4	0.24
miR-205	31.7 ± 0.7	32.5 ± 0.6	32.1 ± 0.3	32.1 ± 0.5	0.77

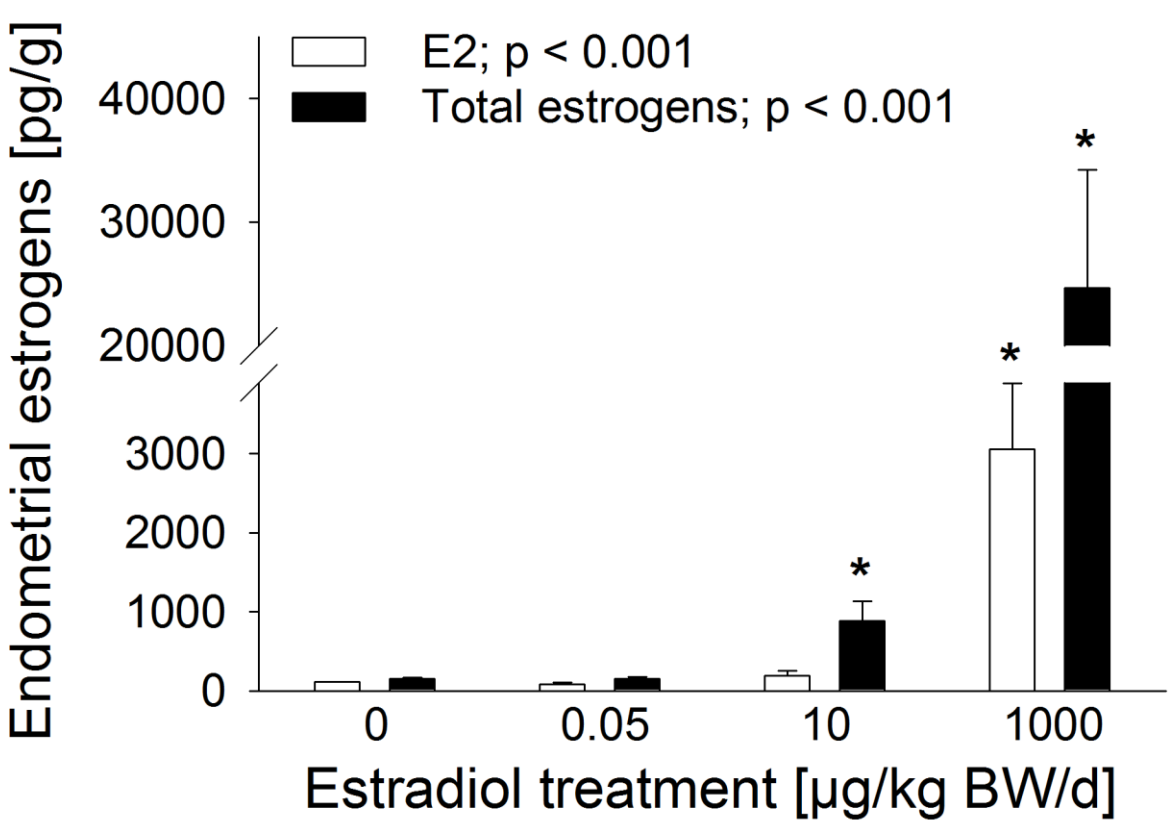

Figure 1

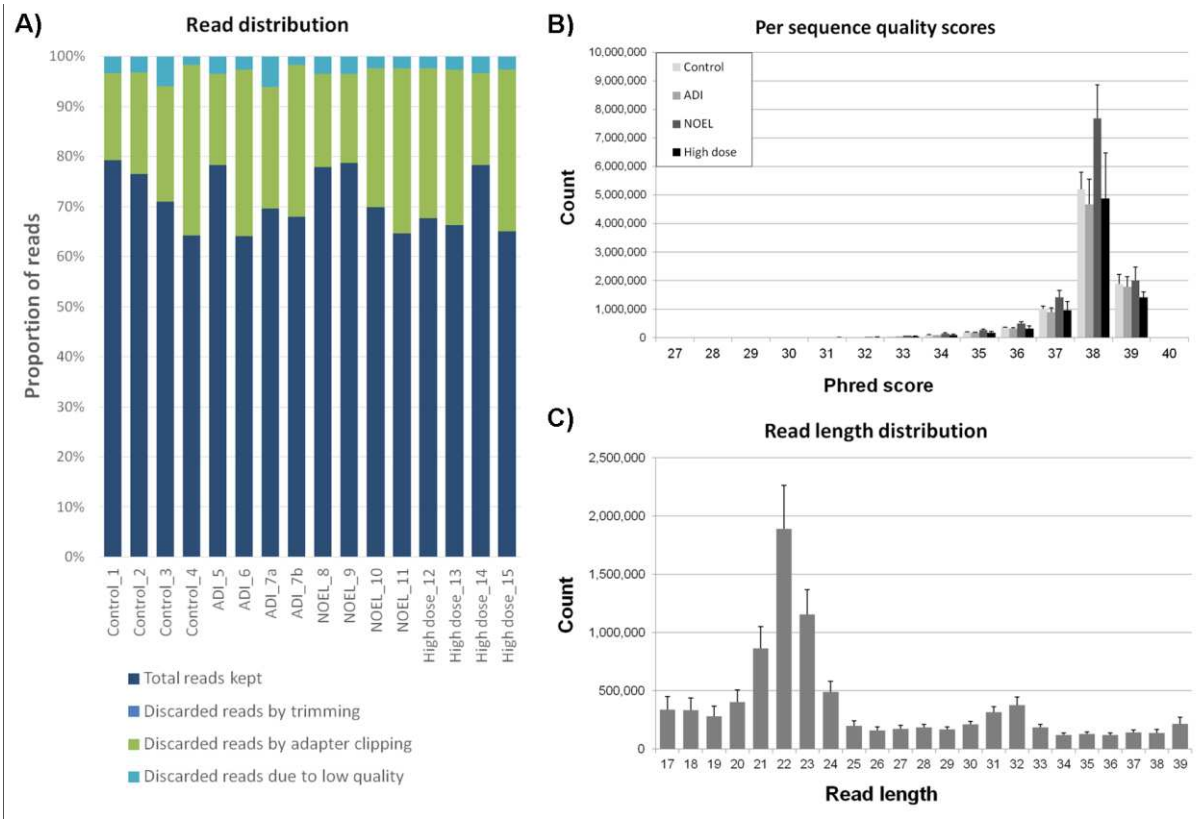

Figure 2

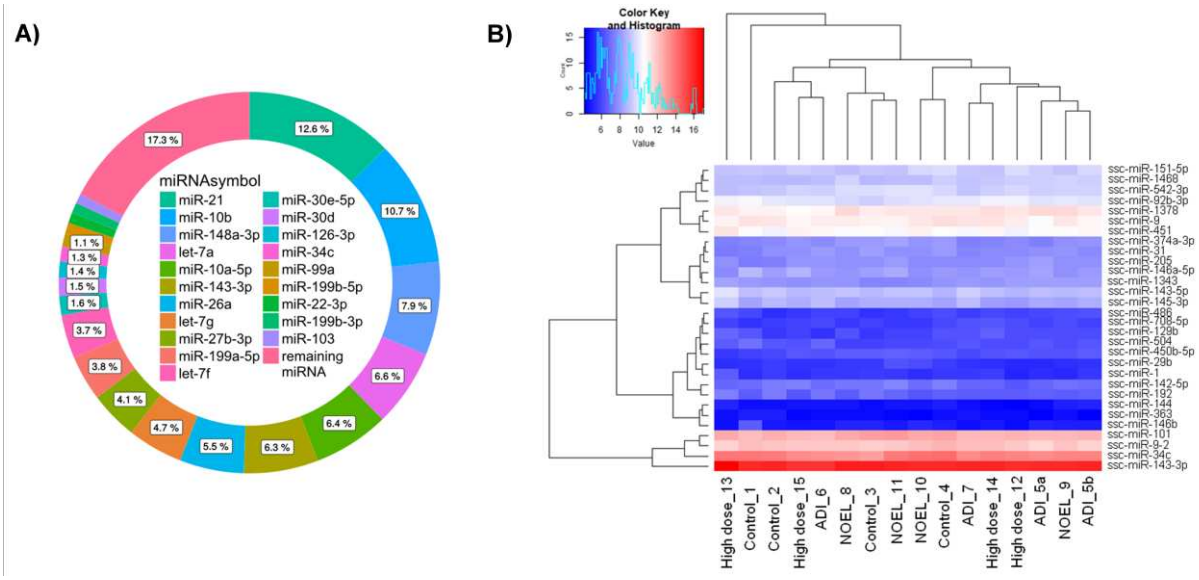

Figure 3

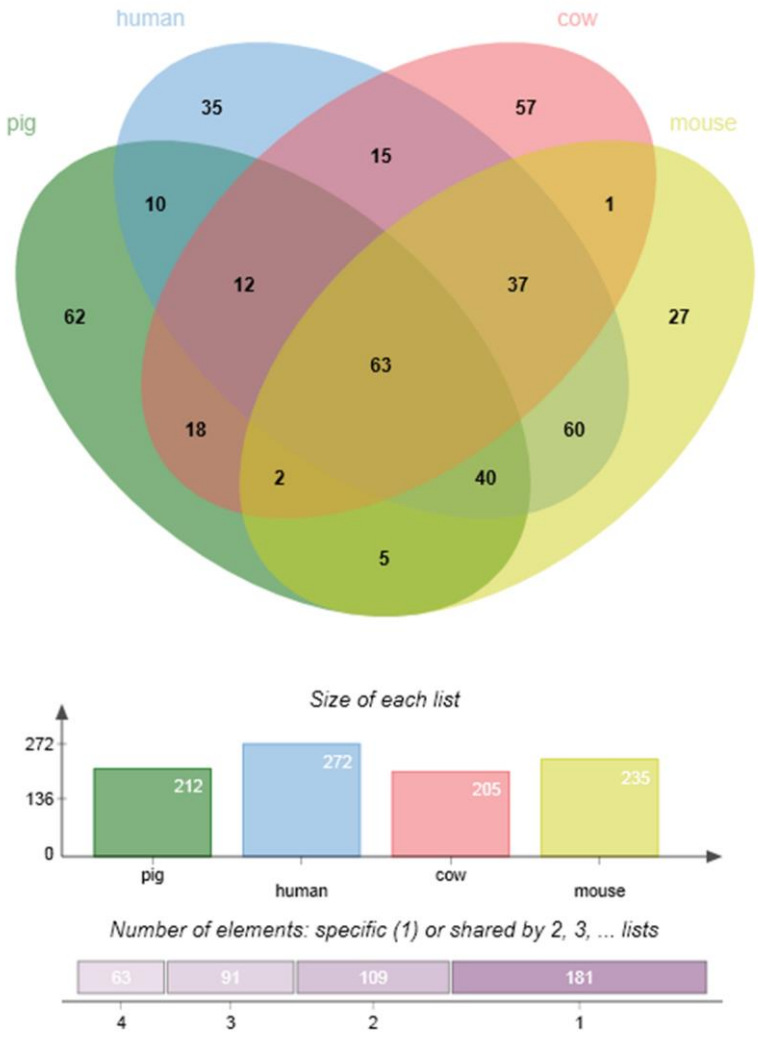

Figure 4